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(54) Title: QUANTITATIVE ANALYSIS OF GENE EXPRESSION

(57) Abstract

A method is provided for determining the level of expression of a gene of interest, or panel of genes of interest, in a biological sample. The method involves preparing a standard curve for each gene of interest by assaying a dilution series prepared from a total RNA in a reverse transcriptase–5'–exonuclease PCR amplification assay. A threshold cycle (Ct) for each member of the reverse transcribed–amplified is determined and plotted versus the log of the amount of total RNA in dilution series. The plot is used to determine an RNA equivalent from which the normalized RNA equivalent for the gene of interest in the sample is determined. The assay can be used to determine the effect of a treatment of the sample on the level of expression of a gene or interest or panel of genes of interest.

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QUANTITATIVE ANALYSIS OF GENE EXPRESSION

TECHNICAL FIELD

This invention relates generally to quantitative reverse transcriptase-polymerase chain reaction ("PCR") assays. More particularly, the invention relates to reverse transcriptase-PCR methods using relative RNA standards for quantitating target mRNA in a sample. The invention has applications in quantitative gene expression, wherein determining changes in gene expression provides a measure of biological response or may be of prognostic or diagnostic value.

BACKGROUND OF THE INVENTION

Quantitative measurement of a nucleic acid target (DNA or mRNA) by PCR requires time-consuming post-amplification steps that can introduce uncontrolled variables and risk of carry-over contamination. In order to address these problems, techniques that permit a more direct detection of PCR products have been developed.

The fluorescence energy transfer ("FET") PCR assay uses a nonextendable nucleic acid probe complementary to an internal segment of the target DNA. The probe is labeled with two fluorescent moieties with the property that the emission spectrum of one overlaps the excitation spectrum of the other. As a result, the emission of the first fluorophore is largely quenched by the second fluorophore. The probe is present during PCR and if PCR product is made the probe becomes susceptible to degradation via the inherent 5'-exonuclease activity of DNA polymerase that is specific for DNA hybridized to its complement. Nucleolytic degradation of the probe allows the two fluorophores to separate in solution, which reduces quenching and increases the intensity of the emitted light. No further post-amplification processing is required when samples are measured in a fluorescent plate reader.

Typically, the detection of DNA by quantitative PCR and mRNA by quantitative reverse transcriptase-polymerase chain reaction ("RT-PCR") requires the use of an internal control that is coamplified with the target sequence. So that it may be distinguished from the target sequence, the internal control can be a scrambled internal sequence, a mutation of the target amplicon, a deletion or insertion of a sequence within the target amplicon, or a target primer sequence spliced onto a nonhomologous DNA sequence. However, differences in amplification efficiency between control and experimental nucleic acids can lead to significant differences in the amounts of their PCR products.

More recently, quantitative PCR methods, using FET PCR to follow the amount of PCR product accumulated in real time, have been developed that use the number of cycles required to reach a predetermined amount of PCR product as a measure of the initial concentration of target nucleic acid. However, these assays also require the use of a coamplified control and, therefore, assumptions about relative amplification efficiency in different samples and between the samples and the control during the exponential amplification phase of PCR.

In addition, it is time-consuming and cumbersome to screen a sample for the level of expression of a number of genes of interest to determine a "profile" of the expression activity under normal conditions or in response to a stimulus. One of the principal difficulties in the approach to profiling gene expression has been in designing readily accessible techniques to quantitatively measure the expression of a panel of genes that have widely varying levels of abundance.

SUMMARY OF THE INVENTION

There is a need in the art for an assay that will allow a determination of the level of expression of a gene of interest without the problems inherent in previous methods. In addition, there is a need in the art for a method that would easily enable testing a gene or groups of genes of interest for changes in expression in response to a stimulus. Such methods should be amenable to small tissue samples, that would eventually allow direct comparison between serial experimental samples from the same subject, controlling for potential background genetic variation, as well as analysis of clinical specimens. The method disclosed and claimed herein for measuring normalized RNA equivalents satisfies these criteria for gene expression profiling.

Accordingly, it is an object of the invention to provide a quantitative RNA profiling method based on the 5'-exonuclease assay for reverse transcriptase polymerase chain reaction ("RT-PCR").

It is another object of the invention to provide a method for determining a quantitative measure of the expression of a gene of interest by determining the relative level in a sample of a target mRNA encoded by the gene of interest.

It is still another object of the invention to provide a method for determining the effect of a stimulus on the quantitative measure of the expression of the gene in response to a stimulus.

It is yet another object of the invention to provide a method for determining a profile of the levels of expression of a panel of genes in a sample.

It is yet a further object of the invention to provide a method for determining the effect of a stimulus on the profile of the levels of expression of genes in a sample.

In one embodiment, then, a method is provided for determining a quantitative measure of the expression of a gene of interest in a biological sample by determining a normalized RNA equivalent for the gene of interest in the sample. The method comprises, first, preparing a standard curve for the gene of interest using a reverse transcriptase-5'-exonuclease polymerase chain reaction (RT-PCR). The standard curve is prepared by a method comprising (i) preparing a reverse transcript standard dilution series from a total RNA extract having a known total RNA concentration, (ii) amplifying each member of the reverse transcript standard dilution series using a 5'-exonuclease PCR assay, wherein the components of the assay comprise a forward primer, a reverse primer, a nonextendable FET hybridization probe and a thermostable DNA polymerase, and wherein the primers and the probe are capable of specifically hybridizing to and forming a stable hybrid duplex with a segment of the reverse transcript of mRNA encoded by the gene of interest, or its complement, the primers are capable of priming an extension reaction, and the DNA polymerase is capable of catalyzing a primer extension reaction and has 5'-exonuclease activity, (iii) monitoring fluorescence intensity from the 5'-exonuclease PCR assay in real time during each cycle of the PCR for each member of the reverse transcript standard dilution series, (iv) determining a threshold cycle (Ct) for each member of the reverse transcript standard dilution series, (v) determining an RNA equivalent from a plot of Ct versus the log of the amount of total RNA in each member of the reverse transcript standard dilution series, and (vi) normalizing the RNA equivalent to provide a normalized RNA equivalent standard curve for the gene of interest. The normalized RNA equivalent for the gene of interest is determined by assaying the sample in the RT-PCR and comparing the results therefrom with the standard curve.

In another embodiment of the invention, a method is provided for determining the effect of a treatment on the quantitative measure of the expression of a gene of interest in a sample. The method comprises preparing a standard curve for the gene of interest using the aforementioned RT-PCR assay. The normalized RNA equivalent for the gene of interest in a first untreated sample and a second treated sample is determined by assaying the

first and second samples in the RT-PCR and comparing the results obtained therefrom with the standard curve.

In yet another embodiment of the invention, a method is provided for determining a quantitative measure of the expression of a panel of genes of interest in a sample. The method comprises preparing a standard curve for each of the genes of interest using the aforementioned RT-PCR assay and a separate and distinct set of a forward primer, a reverse primer and a hybridization probe for each gene of interest. The normalized RNA equivalents for the genes of interest in the sample are determined by assaying the sample in the RT-PCR for each gene of interest and comparing the results obtained therefrom with the standard curve for each respective gene of interest.

It is a further object of the invention to provide a method for determining the effect of a treatment on a quantitative measure of the expression of a panel of genes of interest in a first untreated sample and a second treated sample. The method comprises preparing a standard curve for each gene of interest using the aforementioned RT-PCR assay. The normalized RNA equivalent for each gene of interest in the first and second samples is determined by assaying the samples in the RT-PCR and comparing the results obtained therefrom with the standard curve for each respective gene of interest.

In yet a further embodiment, the invention is directed to kits for conducting the aforementioned method comprising (a) a forward primer, a reverse primer and a hybridization probe, which primers and probe are capable of specifically hybridizing to the reverse transcript of the target mRNA molecule or its complement and of forming a stable hybrid duplex with a segment thereof, the primers also being capable of priming an extension reaction, (b) a reverse transcriptase, (c) a thermostable DNA polymerase, (d) instructions for conducting the method and, optionally (e) other reagents necessary for conducting the reverse transcription and/or the PCR amplification reactions.

These and other objects, embodiments and features of the invention will become apparent to those skilled in the art upon reading the following disclosure and description of the invention.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A is a graphical illustration of the increase in fluorescence intensity from hybridization probe degradation, expressed as ΔRn, as a function of PCR cycle number for a four-fold serial dilution series of a total RNA extract from mouse ventricle using forward and reverse primers and a hybridization probe specific for cardiac ankyrin repeat protein. The

concentrations of total RNA in ng/reaction tube run in duplicate were 400 (closed circles), 100 (closed triangles), 25 (closed squares), and 6.25 (closed diamonds). The PCR threshold cycle at which each PCR amplification reaction reaches ten times the standard deviation of the fluorescence baseline (Ct) is indicated by an arrow. FIG. 1B is a graphical illustration of the Ct determined from FIG. 1A plotted against the amount of total RNA in ng/reaction on a log scale. The linear regression equation determined for the points plotted in FIG. 1B is $y=24.90-3.36\ log(x)$, R=0.997.

FIG. 2A and FIG. 2B are graphical illustrations of the comparative molecular phenotype for developmental and pressure overload cardiac hypertrophy (POL). Differential gene expression values for neonate versus adult (FIG. 2A) and POL versus adult (FIG. 2B) are plotted as mirror image bar graphs with induction in open bars and repression in striped bars.

DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, cardiovascular physiology, and pharmacology, that are within the skill of the art. Such techniques are explained fully in the literature. *See*, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); *DNA Cloning*, Vols. I and II (D.N. Glover ed. 1985); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Transcription and Translation* (Hames et al. eds. 1984); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller et al. eds. (1987) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); Scopes, *Protein Purification: Principles and Practice* (2nd ed., Springer-Verlag); and *PCR: A Practical Approach* (McPherson et al. eds. (1991) IRL Press).

All patents, patent applications and publications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, for example, reference to "a primer" includes two or more such primers, reference to "a probe" includes more than one such probe, reference to "a gene of interest" includes two or more such genes of interest, a "treatment" includes refers to one or more such treatments, and the like.

A. DEFINITIONS

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The use of the phrase "standard curve" is intended to mean a mathematical transformation of a data obtained from a graphical depiction of the results of an RT-PCR assay in which known quantities of RNA, preferably serial dilutions thereof, are assayed to compare the results obtained thereby with the results obtained for a sample containing an unknown quantity of target RNA for the purpose of quantitating the amount of target RNA in a sample. A standard curve is developed for each gene of interest from a total RNA extract. The total RNA extract is preferably obtained from the same tissue and/or species from which the unknown sample is obtained. The total RNA extract may be fresh or frozen and, if frozen, may be stored as an extract or as a tissue sample that may be extracted immediately before use. The phrase "standard curve" may also be used to refer to a total RNA or cDNA dilution from which the graphical data is obtained.

By "PCR threshold cycle" or "Ct" is intended the PCR cycle at which each PCR amplification reaction reaches a significant threshold, e.g., ten times the standard deviation of the fluorescence baseline.

A "dilution series" is a set of dilutions of total RNA recovered from a biological sample or cDNA prepared from the total RNA used as standards from which a standard curve is prepared for each gene of interest; the results of the reverse transcription and/or PCR amplification of the dilution series defines a standard curve for each gene. The members of the series may be prepared by diluting the RNA or cDNA in a serial or a parallel manner. The RNA or cDNA standards can be from control, sham-operated, treated, or other category of biological sample that contains and/or expresses the gene of interest.

The term "control total RNA" is intended to mean total RNA extracted from a sample that is used as a measure of the baseline condition of a sample. The term "treated total RNA" is intended to mean total RNA extracted from a sample that has been subjected to a treatment or intervention that is being tested for its effect on the expression of the gene of interest.

The phrase "RNA equivalent" is used to refer to the measured level of target RNA in a sample relative to the total RNA standard curve determined for the gene of interest using a reverse transcriptase-5'-exonuclease assay, as described below. The phrase "normalized RNA equivalent" refers to a RNA equivalent that has been normalized for the amount of RNA in each reaction vessel in the 5'-exonuclease assay.

The term "treatment" is used to intend any manipulation of a biological sample or of a subject from which a biological sample is derived, including but not limited to a physiological treatment, e.g., the induction of cardiac pressure overload hypertrophy by aortic constriction, a pharmacological treatment such as exposure to a chemical agent having or suspected of having a physiological, biochemical, therapeutic or toxic effect on the biological sample or subject, onset of a pathological state, such as the onset or progression of a disease, e.g., cancer, heart failure, or the like, surgical treatment including sham operations, genetic treatment such as gene therapy or genetic manipulations to produce a knockout cell or animal, or a transgenic cell or animal, combinations of any of the aforementioned treatments, an adaptive response to any of the above treatments, or the like.

As used herein, the terms "polynucleotide" and "oligonucleotide" shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), to any other type of polynucleotide which is an N- or C-glycoside of a purine or pyrimidine base, and to other polymers containing nonnucleotidic backbones, for example, polyamide (e.g., peptide nucleic acids (PNAs)) and polymorpholino (commercially available from the Anti-Virals, Inc., Corvallis, Oregon, as Neugene™ polymers), and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. There is no intended distinction in length between the term "polynucleotide" and "oligonucleotide," and these terms will be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include, for example, 3'-deoxy-2',5'-DNA, oligodeoxyribonucleotide N3'→P5' phosphoramidates, 2'-O-alkyl-substituted RNA, double- and single-stranded DNA, as well as double- and single-stranded RNA, DNA:RNA hybrids, and hybrids between PNAs and DNA or RNA, and also include known types of modifications, for example, labels which are known in the art, methylation, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), and with positively charged linkages (e.g., aminoalklyphosphoramidates, aminoalkylphosphotriesters), those containing pendant moieties, such as, for example, proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron,

oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide or oligonucleotide.

The term "primer" is used to refer to a oligonucleotide composed of DNA, and/or RNA, and/or synthetic nucleotide analogs, and is capable of acting as a point of initiation of synthesis along a complementary polynucleotide molecule under conditions suitable for synthesis of a primer extension product. Such conditions include the presence of four different deoxyribonucleotide triphosphates and at least one polymerization-catalyzing agent such as a reverse transcriptase or a DNA polymerase. The deoxyribonucleotides are present in a suitable buffer, including necessary cofactors, at a suitable temperature. Preferably a primer is a single-stranded oligonucleotide.

As used herein, the term "target region" or "target nucleotide sequence" refers to a region contained within the gene of interest, the mRNA encoded by the gene of interest, a reverse transcript of the mRNA encoded by the gene of interest, or the complement of the gene of interest or the mRNA encoded by the gene of interest, that is to be amplified and/or detected. The term "target sequence" refers to a sequence with which a probe will form a stable hybrid under desired conditions.

As used herein, the term "hybridization probe" refers to a structure comprised of a polynucleotide, as defined above, that contains a nucleic acid sequence complementary to a nucleic acid sequence present within the gene of interest, a reverse transcript of the mRNA encoded by the gene of interest, or the complement of the gene of interest or the mRNA encoded by the gene of interest. The hybridization probe may be composed of DNA, and/or RNA, and/or synthetic nucleotide analogs. In addition, a probe is optionally comprised of a 3'-phosphate such that the probe does not serve as an extension primer for DNA polymerase or reverse transciptase. A "probe" also comprises a detectable label which, when the probe is intact and undegraded does not produce a signal and when released from its complement by 5'-exonuclease digestion, gives rise to a detectable signal. The detectable label typically consists of two different fluorescent dyes. One dye is a reporter dye and the other is a quenching dye. When the probe is intact, fluorescent energy transfer occurs and the reporter dye fluorescence emission is absorbed by the quenching dye. During the extension phase of the PCR cycle, the fluorescent hybridization probe is cleaved by the 5'-3' nucleolytic activity of the DNA polymerase. On cleavage of the probe, the reporter dye emission is no longer

transferred efficiently to the quenching dye, resulting in an increase of the reporter dye fluorescence emission.

It will be appreciated that the hybridizing sequences need not have perfect complementarity to provide stable hybrids. In many situations, stable hybrids will form where fewer than about 10% of the bases are mismatches, ignoring loops of four or more nucleotides. Accordingly, as used herein the term "complementary" refers to an oligonucleotide that forms a stable duplex with its "complement" under assay conditions, generally where there is about 90% or greater homology.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a subject, including but not limited to, for example, plasma, serum, spinal fluid, semen, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, biopsies and also to samples of *in vitro* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components). Preferred uses of the present method are in detecting and/or quantitating gene expression as follows: genetic manipulations such as gene therapy, production of a knockout cell or animal and production of a transgenic cell or animal; surgical animal models such as models of myocardial infarction or pressure overload hypertrophy; clinical samples such as biopsies obtained during clinical trials or for research purposes, e.g., cancerous breast tissue; and responses of cultured cells and/or tissues to chemical treatment.

"Optional" or "optionally" means that the subsequently described circumstance may or may not occur, and that the description includes instances in which said circumstance occurs and instances in which it does not. For example, the phrase "optionally including a ceramic powder" means that a ceramic powder may or may not be present and that the description includes both the instance when the ceramic powder is present and the instance when the ceramic powder is not present.

The present invention used quantitative reverse transcriptase polymerase chain reaction using real time detection and the 5'-exonuclease assay to determine the level of expression of a target gene. The 5'-exonuclease assay for RT-PCR, otherwise known as the 5'-nuclease assay, the nick translation assay, or the like, has been described in, for example, Holland et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:7276-7280, Gibson et al. (1996) *Genome Res.* 6:995-1001 and Heid et al. (1996) *Genome Res.* 6:986-994. Briefly, the 5'→3' exonuclease assay for detecting PCR products uses a

nonextendable oligonucleotide hybridization probe. The probe is doubly labeled to monitor the results of the assay. The probe is labeled with a reporter flourescent dye, i.e., a fluorescent phosphoramidite, at the 5' end, e.g., 6-carboxy-fluorescein ("FAM"), 2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein ("JOE"), and tetrachloro-6-carboxy-fluorescein ("TET"), and a quencher fluorescent dye, e.g., 6-carboxy-tetramethyl rhodamine ("TAMRA"). The labeled probe is designed to anneal to a region of one strand of the PCR product downstream from one of the PCR primers. The 3' end of the probe is blocked from extension by a 3'-phosphate. The probe is included with the other PCR reagents, e.g., dNTPs, and, when annealed, is degraded by the 5'→3' exonuclease activity inherent in the DNA polymerase. When the probe is intact the reporter dye emission is quenched owing to the physical proximity of the reporter and quencher fluorescent dyes. During the extension phase of the PCR cycle, however, the nucleolytic activity of the DNA polymerase cleaves the hybridization probe and releases the reporter dye from the probe.

The resulting relative increase in reporter fluorescent dye emission is monitored in real time during PCR amplification. A comparison of the amount of reporter dye emission (R) with a passive reference dye, e.g., 5-carboxy-X-rhodamine (ROX) (P) during the PCR amplification generates a ΔRn value (R/P). The ΔRn value reflects the amount of hybridization probe that has been degraded. An exponential function may be fit to the mean ΔRn values of, typically, the last three data points of each PCR extension cycle, generating an amplification plot. A relative fluorescent emission threshold is set based on the baseline of the ΔRn during the first 10-15 cycles (*see* Heid et al. (1996) *Genome Res.* 6:986-994). The cycle at which each PCR amplification reaches a significant threshold, e.g., ten times the standard deviation of the baseline, the Ct is calculated. The Ct is proportional to the number of target copies present in the sample. Heid et al. (1996), *supra*. Thus, the Ct value is a quantitative measurement of the copies of the target in any sample.

Rapid measurement of relative changes in gene expression levels can be achieved using the invention disclosed and claimed herein by determining target gene RNA equivalent values using total RNA standard curves. Thus, it is not necessary to use an internal control, i.e., a set of reactions to which a known number of copies of a co-amplified polynucleotide is added, to determine the number of copies of the unknown target gene RNA. The level of expression of the target gene is determined by calculating the amount of target gene RNA relative to the total RNA used for the standard curve, i.e., the relative RNA equivalent.

A typical RT-PCR assay is conducted as follows. Total RNA is extracted from a biological sample. If an assay includes a control biological sample and a biological sample subject to an experimental treatment, stimulus or other intervention, control total RNA is extracted from the control sample and treated total RNA is extracted from the treated sample.

Cells or homogenized tissue samples are lysed by methods that disrupt the cells and, preferably, inactivate ribonucleases. RNAses are inactivated by 4 M guanidinium thiocyanate and reducing agents such as β-mercaptoethanol. Such a combination of reagents can be used to isolate intact RNA from tissues that contain RNAse activity. *See* Sambrook et al., pages 7.6-7.11.

Total RNA may be extracted from using any method known in the art. For example, total RNA may be isolated using the guanidinium thiocyanate/cesium chloride method described by Glisin et al. (1974) *Biochemistry* 13:2633, Ullrich et al. (1977) *Science* 196:1313, and Chomczynski et al. (1987) *Anal. Biochem.* 162:156 or by the guanidine HCl/organic solvents method described in Strohman et al. (1977) *Cell* 10:265 and McDonald et al. (1987) *Meth. Enzymol.* 152:219.

Alternatively, RNA may be extracted using any of a number of commercially available kits, for example, RNA-STAT-60 (Tel-Test, Inc., Friendswood, TX), the RNeasy total RNA extraction kit (Qiagen), Tripure (Boehringer Mannheim Biochemicals, Indianapolis, IN), Trizol (GIBCO Laboratories, Gaithersburg, MD), and TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH).

Dilutions of the total RNA extracts are used to prepare a standard curve. A new standard curve is generated each time a gene of interest is assayed using forward and reverse primers and a hybridization probe specific for the gene of interest. Typically, reactions are conducted using between about 0.1 ng total RNA per reaction to about 5000 ng RNA per reaction, preferably between about 5 ng RNA per reaction tube to about 1000 ng RNA per reaction tube. The range of total RNA extract per reaction can be adjusted depending on the relative abundance of the target gene and the amount of test RNA per reaction. Thus, for relatively abundant genes about the amount of total RNA extract added per reaction tube will be at the lower range of amounts while for more rare transcripts the amount of total RNA extract will be at the higher range of amounts.

In addition to running a total RNA standard curve for each gene of interest, a standard curve is optionally run for a housekeeping gene to normalize for the amount of total RNA added to each reaction. A "housekeeping gene" is a gene the expression of which is substantially the same from sample

to sample or from tissue to tissue, or one that is relatively refractory to change in response to external stimuli. A housekeeping gene can be any RNA molecule other than that encoded by the gene of interest that will allow normalization of sample RNA or any other marker that can be used to normalize for the amount of total RNA added to each reaction. For example, the GAPDH gene, the G6PD gene, the β-actin gene, ribosomal RNA, 36B4 RNA, or the like, may be used as a housekeeping gene. Thus, the dilutions of total RNA extract used to prepare the standard curve for the expression of the target gene may also be assayed for the expression of, for example, the GAPDH gene. The PCR threshold cycle is determined for each of the total RNA standard concentrations and, when assayed, for the housekeeping gene. The Ct values thus determined are plotted against the $\log_{10}$ of the concentration of total RNA in the corresponding reaction to obtain a standard curve. The equation of this curve is used to determine equivalent values of input RNA for normalization or to determine RNA equivalents.

The total RNA in each reaction tube is reverse transcribed using any naturally occurring or recombinant enzyme that has reverse transcriptase activity, e.g., AMV reverse transcriptase, MMLV reverse transcriptase, *Tth* DNA polymerase, and the like. The reverse transcripts are then amplified in the presence of target-specific forward and reverse primers and a target-specific hybridization probe using PCR thermocycling methods well known in the art. The housekeeping gene reverse transcripts are amplified using forward and reverse primers and hybridization probes specific to the housekeeping gene using PCR thermocycling method.

The 5'-exonuclease PCR assay uses any naturally occurring or recombinant enzyme that has DNA polymerase activity and 5'-exonuclease activity and that is thermally stable. A thermostable DNA polymerase is a DNA polymerase that, at elevated temperatures, maintains the ability to catalyze the addition of deoxyribo-nucleotides to an oligonucleotide primer based on a template DNA sequence. Examples of such thermostable DNA polymerases include: *Taq* DNA polymerase (*Thermus aquaticus*); *Tth* DNA polymerase (*Thermus thermophilus* HB8; and *Tfl* DNA polymerase (*Thermus flavus*).

More particularly, PCR employs short oligonucleotide primers (generally 10-20 nucleotides in length) that match opposite ends of a desired sequence within a DNA molecule. The initial template can be either RNA or DNA. If RNA is used, it is first reverse transcribed to cDNA. The cDNA is then denatured, using well-known techniques such as heat, and appropriate oligonucleotide primers are added in molar excess.

Primers hybridize to a complementary target nucleotide sequence and primer extension is catalyzed using DNA polymerase in the presence of deoxynucleotide triphosphates or nucleotide analogs. The resulting product includes the respective primers at their 5'-termini, covalently linked to the newly synthesized complements of the original strands. The replicated molecule is again denatured, hybridized with primers, and so on, until the product is sufficiently amplified. Such PCR methods are described in *e.g.*, U.S. Patent Nos. 4,965,188; 4,800,159; 4,683,202; 4,683,195; incorporated herein by reference in their entireties.

The increase in reporter dye fluorescence emission is monitored during the PCR amplification in real time and used to calculate the ΔRn. "ΔRn" is the fluorescence signal increase due to target or housekeeping gene reverse transcript amplification and is calculated by subtracting the background fluorescence: $\Delta R = (Rn^+)-(Rn^-)$, wherein Rn^+ is ratio of the emission intensity of the reporter dye to the emission intensity of the passive reference dye during amplification in the presence of target reverse transcript and Rn^- is ratio of the emission intensity of the reporter dye to the emission intensity of the passive reference dye during amplification in the absence of target reverse transcript. The ΔRn is plotted versus the amplification cycle number for each of the total RNA dilution standards and for each of the housekeeping gene dilution standards. A Ct is determined for each of the total RNA dilution standards and for the housekeeping gene dilution standards.

A standard curve is prepared for each gene of interest and, optionally, for a housekeeping gene, by plotting the Ct against the $\log_{10}$ of the concentration of total RNA for the dilution series to yield a typically linear standard curve. The standard curve may be fitted to a linear equation $Y=MX+B$ for each gene of interest and $y=mx+b$ for the housekeeping gene in which Y and y are the Ct, M and m are the slopes of the lines and B and b are the values of Ct where the fitted lines intercept the ordinates.

Normalized RNA equivalents for unknown control and/or treated samples are calculated from the plots of Ct vs [RNA] as follows. The unknown control and/or treated sample RNA extract is assayed in the RT-PCR assay using forward and reverse primers and a hybridization probe specific for each gene of interest. Preferably, the sample RNA extract is assayed in replicate for each gene of interest. The sample RNA equivalent is calculated by solving the linear equation for X, namely, $X=(Y-B)/M$ for the standard curve determined for the gene of interest; X is then normalized to the amount of total RNA in the unknown sample by solving for x, namely, $x=(y-b)/m$ for the RNA equivalent based on the housekeeping gene ("*hg*"). Thus, for example, in an assay in

which 0.5 µg/reaction total RNA in the unknown test control sample is added, the RNA equivalent for an unknown sample may be calculated as follows:

Unknown 1 (e.g., control sample)

$$\text{RNA equivalent}_1 = \frac{10^{((Ct_1 - B)/M)} \times 0.5\mu g/rxn}{10^{((Ct_1(hg) - b)/m)}}$$

In an assay in which 0.5 µg/reaction total RNA in the unknown test treated sample is added, the amount of unknown RNA equivalent may be calculated as follows:

Unknown 2 (e.g. treated sample)

$$\text{RNA equivalent}_2 = \frac{10^{((Ct_2 - B)/M)} \times 0.5\mu g/rxn}{10^{((Ct_2(hg) - b)/m)}}$$

In order to compare the amount of mRNA expressed from the gene of interest in response to the treatment, RNA equivalent$_2$ is compared with RNA equivalent$_1$.

Forward and reverse primers and a hybridization probe capable of specifically hybridizing to the mRNA molecule encoded by the gene or genes of interest, or the complement thereof, and capable of forming a stable hybrid duplex with a segment thereof, the primers also being capable of priming an extension reaction, a reverse transcriptase, and/or a thermostable DNA polymerase can be provided in diagnostic kits. The kit also may contain other suitably packaged reagents and materials needed or desirable for the particular assay protocol, for example, reagents for extracting RNA from a sample, dNTPs as well as instructions for performing the assay.

It is contemplated that reagents employed in the above kit can be provided in one or more containers such as vials or bottles, with each container containing a separate reagent such as a reverse transcriptase, a dNTP or a cocktail of dNTPs, or a thermostable DNA polymerase employed in the assay. Other components such as buffers, controls, and the like, known to those of ordinary skill in art, may be included in such test kits. The kits will also include instructions for the use thereof.

An analytical RT-PCR assay can be used to measure quantitative changes in gene expression. This method is sensitive, requires very little tissue sample, and is adaptable to rapidly testing new candidate genes. In contrast to previously reported methods, the present method does not require cloned

cDNA, or a fragment thereof, or an artificial transcript prepared from cDNA for quantitative calibration of the assay. Furthermore, previously reported methods use relatively pure or purified RNA standards. By contrast, the present assay uses total RNA as a standard; the use of such a more complex standard more closely reflects the composition of an unknown sample. Moreover, the assay requires only knowledge of the DNA sequence complementary to the mRNA encoded by the gene of interest to enable PCR primer and probe design. In addition, the assay method disclosed and claimed herein can be used to screen numerous candidate genes for changes or differences in expression.

Gene expression data on a very large number of genes is currently being gathered using microarray-based technologies and expressed sequence tag approaches. Application of these technologies to expression analysis in *in vitro* and *in vivo* models of development and pathophysiology will identify subsets of genes uniquely associated with each biological system. Application of analytical PCR should provide an accessible means to rapidly focus on select markers that represent a fingerprint of the molecular phenotype.

The ability to quantitatively measure changes in gene expression with small amounts of total RNA means that this analytical PCR assay will be widely applicable to testing human biopsy samples. The amount of material typically obtained in a biopsy does not support a microarray-based expression assay; however, such sample is sufficient to be used in the RT-PCR disclosed and claimed herein. The ability to make these measurements in human samples will provide a means to identify diagnostic molecular markers associated with disease progression and prognosis, and to determine the effect of treatment strategies. This data will also provide an important link to animal models of human disease.

For example, when challenged by a variety of neurohumoral agents, mechanical stimuli, and cellular injury, the adult heart responds by cardiac muscle cell hypertrophy. At a cellular level, the hypertrophic response is characterized by an increase in the size of individual myocytes without a concomitant change in cell number. Although initially compensatory, a pathological transition can occur accompanied by cardiac muscle dysfunction and overt heart failure. A determination of the patterns of gene expression observed in different models of hypertrophy would provide clues to distinguish between adaptation and dysfunction. Such a determination would permit the identification of the pathways which activate different forms of cardiac hypertrophy, and which ultimately drive the pathological phenotype of cardiac chamber dilation and failure.

One of the most conserved features of cardiac hypertrophy is the re-activation of genes that are normally limited in their expression to the embryonic window of heart development. For example, the atrial natriuretic peptide (ANP) gene is expressed in both the embryonic atrial and ventricular chambers, but is down-regulated in the ventricle during normal post-natal development. However, ventricular ANP gene expression is rapidly induced by greater than 10-fold in response to hemodynamic loading, a response common to all vertebrate species thus far examined. These findings, together with data on a number of other genes have led to the hypothesis that there are common pathways involved in the regulation of developmental gene expression and in the setting of adult cardiac hypertrophy in response to increased load. These shared pathways would then result in part in a shared molecular phenotype between the two tissues states. Although widely recognized, this hypothesis has yet to be rigorously tested, as only a few candidate genes have thus far been examined at critical time points of cardiac ventricular embryonic development, normal cardiac post-natal growth, and during cardiac hypertrophy in the adult heart.

Methods for measuring *in vivo* heart function in the mouse, combined with mouse genetics and molecular genetic techniques, offer the opportunity to directly determine the effects of genotype on cardiac physiology. Based on gene knockout and transgenic strategies several mouse models of cardiac pathology have been described, including hypertrophy by cardiomyocyte-specific transgene expression of the α_1B-adrenergic receptor, activated H-ras p21, and activation of gp130 signal transduction. Dilated cardiomyopathy has also been described in MLP and MnSOD knockout mice. While these various mouse models have functional and pathological abnormalities similar to human heart failure, the extent to which these genetic modifications recapitulate the molecular events involved in the initiation and progression of cardiac muscle disease is unknown. The missing aspect of these studies is the monitoring of changes in the gene expression profile, the molecular phenotype, based on the genotype of the mouse, and relating how this data compares to human cardiac physiology, pathology, and molecular phenotype.

Using the method disclosed herein, a gene expression profile for the mouse myocardium in normal growth and adaptive hypertrophy was developed. The present RT-PCR assay was applied to a panel of 29 genes in tissue samples derived from three stages of cardiac growth; late stage embryonic ventricular chamber development, normal post-natal cardiac growth, and postnatal hypertrophy following pressure overload from aortic coarctation. Using this approach, evidence is provided that divergent molecular profiles exist for

each one of these distinct stages of ventricular chamber growth. Furthermore, new classes of molecules have been identified to be selectively regulated in each of these settings, suggesting their particular potential importance in differential features of these defined stages of normal and pathological cardiac growth and hypertrophy.

B. Experimental

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of synthetic organic chemistry, biochemistry, molecular biology, and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins, eds., 1984); and the series, *Methods in Enzymology* (Academic Press, Inc.).

It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, that the description above as well as the examples which follow are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

In the following examples, efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental error and deviation should be accounted for. Temperature is always given in degrees C and, unless otherwise indicated, pressure is at or near atmospheric.

SYNTHESIS OF ROX STANDARD. A fluorescent standard 5'-TTT-TTT-(LAN-ROX)-p3', which is included in each PCR reaction, was prepared as follows: "ROX", 5-ROX, SE (5-carboxy-X-rhodamine, succinimidyl ester) was purchased from Molecular Probes, Inc. (Eugene, OR). "LAN", the linker arm nucleotide (amino-modifier C6dT), and 3'-Phosphate-CPG (solid phase support), 46 μm/g, were purchased from Glen Research (Sterling, VA). Synthesis was carried out on an ABI 394 DNA Synthesizer at 10 μm scale according to the following steps: (1) LAN was coupled to the 3'-Phosphate-CPG; (2) T6 Phosphoramidite was coupled to the LAN-3'-Phosphate-CPG; (3) cleavage of the oligonucleotide from the CPG was carried out at room temperature for 1-2 hours in 20 ml of concentrated NH_4OH; (4) the resultant mixture was vortexed and centrifuged and the supernatant removed and evaporated to yield a residue; and (5) the residue was dissolved in 5 ml of 0.25 M $NaHCO_3$ (adjusted to pH 9.0 with 1N NaOH). 5-ROX, SE dye (25 mg/300 μl DMSO) was added as follows: 50 μl of ROX was added, mixed by

agitation and left in the dark at room temperature for 45 minutes. The 50 µl addition was repeated and left in the dark for 45 minutes. A final 50 µl addition was made and the reaction was incubated overnight at room temperature. The reaction was then divided into 10 aliquots and passed through Pharmacia (Piscataway, NJ) PD-10 columns to remove unreacted dye residue. The desired fractions were combined, evaporated, and the crude product was purified on HPLC followed by polyacrylamide gel electrophoresis purification.

SYNTHESIS OF HYBRIDIZATION PROBES. Probes were either purchased from PE, Applied Biosystems (Foster City, CA) or synthesized as follows: "TAMRA" is 5-TAMRA, SE (5-carboxytetramethylrhodamine-X-rhodamine, succinimidyl ester), was purchased from Molecular Probes, Inc. (Eugene, OR). "LAN" the linker arm nucleotide (Amino-Modifier C6dT), 3'-Phosphate-CPG (Support), 46 µm/g, and "FAM", 5-(5'-fluorescein phosphoramidite) were purchased from Glen Research (Sterling, VA). Synthesis was carried out on an ABI 394 DNA Synthesizer at 0.2 µm scale as follows: (1) LAN was coupled to the 3'-Phosphate-CPG in bulk (approximately 34 µm/reaction); (2) using a 0.2 µm scale, the oligonucleotide was synthesized on the LAN-3'-Phosphate-CPG using standard phosphoramidite chemistry; and (3) FAM was coupled to the completed oligonucleotide (7-10 minute coupling time). Cleavage of the oligonucleotide from the CPG was carried out in the dark at 40°C for 18 hours in 1 ml of concentrated NH_4OH. The mixture was vortexed and centrifuged, and the supernatant was removed and evaporated to yield a residue. To precipitate the product, the residue was dissolved in 1 ml of 1 M NaCl and 12 ml of absolute ethanol was added. The mixture was shaken vigorously and left at -20°C overnight. Next the suspension was centrifuged for 6-7 minutes and the supernatant discarded. The orange pellet was dried gently under vacuum in the dark to remove excess ethanol and the resulting residue was dissolved in 0.5 ml of 0.25 M $NaHCO_3$ (adjusted to pH 9.0 with 1 N NaOH). 5-TAMRA, SE dye (5 mg in 60 µl DMSO) was added as follows: 5 µl of TAMRA was added to the reaction, vortexed and left in the dark at room temperature for 45 minutes. The 5 µl addition was repeated and again incubated for 45 minutes. A final 5 µl addition was made and the reaction was incubated overnight at room temperature. The reaction solution was passed through a Pharmacia PD-10 Column to remove unreacted dye, the desired fraction(s) were collected, combined and evaporated, and the crude product was purified on HPLC followed by polyacrylamide gel electrophoresis purification.

TISSUES AND RNA PREPARATION. For the gender and aging study adult C57Bl/6 mice were obtained from The National Institute for Aging (Bethesda, MD). Mice

were euthanized with CO_2 to effect followed by cervical dislocation. Tissues were removed and snap frozen in liquid nitrogen within five minutes, and stored at -80°C until RNA preparation. Neonatal ventricles were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). Pressure overload hypertrophy (POL) was induced by transverse aortic constriction as previously described by Rockman et al. (1993) *Circulation* 87 Supplement VII:VII-14-VII-21.

RNA was extracted using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions and stored at -80°C at a concentration of 0.5 µg/µl in 10-15 µg aliquots. Probe and primer sets for each gene were designed either with Oligo 5.0 (National Biosciences Inc., Plymouth, MN) or Primer Express® (PE Applied Biosystems, Foster City, CA). The rules for designing probe/primer sets are summarized as follows: (1) maximum amplicon size of 500 bp with a preferred range of 50-150 bp; (2) probes have no G at the 5' end, 30-80% (G + C) content, ≤ 3 consecutive Gs, are based on the strand with more Cs than Gs, do not hybridize/overlap with the forward or reverse primers and have Tm = 68-70°C; (3) primers are designed with Tm = 58-60°C, and the five nucleotides in the 3' end of the primers have only 1 to 2 G+C. The forward and reverse primers and probes used in the examples below are listed in Tables 1A, 1B and 1C, respectively.

Table 1A
Mouse Forward Primers

Gene	Forward primer 5' - 3'	SEQ. ID NO:
ANP	CAT CAC CCT GGG CTT CTT CCT	1
BNP	GCG GCA TGG ATC TCC TGA AGG	2
NPR-A	TGG AGA TCG GGA CAC TGA TTT	3
NPR-B	CAC TTC AAT TGG ACG GCT CG	4
ET-1	GCT GTT CCT GTT CTT CCT TGA TGG	5
ETAR	GTT TAA GCT CTT GGC AGG AC	6
ACE	AGC TGC GAA GGA TCA TCG GAT CTA	7
AT1aR	GCT GGC AGG CAC AGT TAC ATA TT	8
PF2aR	AGC AGC ATA GGC AAG GCA GAT	9
VEGF	AAG GAG AGC AGA AGT CCC ATG A	10
IGF-1	TGT CGT CTT CAC ACC TCT TCT ACC T	11
Skel Actin	AGA CAC CAT GTG CGA CGA AG	12
Card Actin	TAT GCC AAC AAT GTC CTA T	13
VSM Actin	AAA CAG GAA TAC GAC GAA G	14
β Actin	AGA TTA CTG CTC TGG CTC CTA	15
a MHC	CCA ATG AGT ACC GCG TGA A	16
β MHC	ATG TGC CGG ACC TTG GAA	17
MLC2a	AGG CAC AAC GTG GCT CTT CT	18
MLP	AGC AAC CCT TCC AAA TTC TCT	19

TGF β1	TGG AGC TGG TGA AAC GGA AGC	20
Coll III	ATG CAG CCA CCT TGG TCA GTC	21
Fibronectin	GGG GCT GGC GCT GTG ACA ACT	22
CARP	CAA AGT TCC AGT TGT GAA GGA	23
GATA-4	CGA GAT GGG ACG GGA CAC T	24
GATA-6	CCG CGA GTG CGT GAA CT	25
SERCA 2	TCC ATG AGC AAG ATG TTT GTG AA	26
PLBN	CGA TCA CCG AAG CCA AGG TCT C	27
MnSOD	CTG GAG GCT ATC AAG CGT GAC TTT	28
MCAD	TCG GAG GCT ATG GAT TCA AC	29
GAPDH	ATG TTC CAG TAT GAC TCC ACT CAC G	30

Table 1b
Mouse Reverse Primers

Gene	Reverse Primer 5' - 3'	SEQ. ID NO:
ANP	TGG GCT CCA ATC CTG TCA ATC	31
BNP	CCC AGG CAG AGT CAG AAA CTG	32
NPR-A	CTG GCC CTC CGT GGT TAG CAG	33
NPR-B	GCC GCA GAT ATA CAC AAT GCG	34
ET-1	AAA TTC CAG CAC TTC TTG TCT TTT TGG TG	35
ETAR	AGA ATC CAG ATG GAG ACG AT	36
ACE	TTG GCA TAG CTT CGT GAG GA	37
AT1Ar	CGG TAT TCC ATA GCG GTA TAG ACA G	38
PF2aR	CGA CTG GCA AGT TTA TAC AGG	39
VEGF	CAC AGG ACG GCT TGA AGA TGT	40
IGF-1	CCA CAC ACG AAC TGA AGA GCA T	41
Skel Actin	CCG TCC CCA GAA TCC AAC ACA	42
Card Actin	CAC AAT ACG GTC ATC CTG AA	43
VSM Actin	CAG GAA TGA TTT GGA AAG GA	44
β Actin	CAA AGA AAG GGT GTA AAA CG	45
A MHC	ACA GTC ATG CCG GGA TGA T	46
β MHC	CCT CGG GTT AGC TGA GAG ATC A	47
MLC2a	AGC TGG GAA TAG GTC TCC TTC A	48
MLP	AGC TCC CCA TCC TTG TCA GTG	49
TGF β1	GTA GAG TTC CAC ATG TTG CTC CAC A	50
Coll III	AGG CCA GGG TCA CCA TTT CTC	51
Fibronectin	TCT AAC GGC ATG AAG CAC TCA	52
CARP	CAG CCT CCA TTA ACT TCT CCA	53
GATA-4	CTC ACC CTC GGC ATT ACG A	54
GATA-6	CGC TTC TGT GGC TTG ATG AG	55
SERCA 2	TCC CGA ATG ACA GAC ATA ATC TTC T	56
PLBN	GTG GCG GCA GCT CTT CAC AGA	57
MnSOD	ATG TGG CCG TGA GTG AGG TTT C	58

MCAD	CTT TTC AAT GTG CTC ACG AG	59
GAPDH	GAA GAC ACC AGT AGA CTC CAC GAC A	60

Table 1C
Mouse TaqMan Probes

Gene	TaqMan probe 5'FAM - TAMRA	SEQ. ID NO:
ANP	ATT TCA AGA ACC TGC TAG ACC ACC TGG A	61
BNP	TCC TTC GGT CTC AAG GCA GCA CCC TCC	62
NPR-A	CCA CCT CCT GAC ATC CCT AAA TGT GGC T	63
NPR-B	CAG CAA CCT CAG TGT GCA GCA CCA GG	64
ET-1	AGG TTC TTC CAG GTC CAA GCG TTC CTT	65
ETAR	CCA CTG CTC TGT ACC TGT CCA CAC TG	66
ACE	TGA GCA GAA TCT ACT CCA CTG GCA AGG TCT	67
AT1Ar	TAA ATC TCG CCC TGG CTG ACT TAT GCT T	68
PF2Ar	TTG CTC TCC GCA TGG CAA CGT GGA ATC A	69
VEGF	TAC CAG CGA AGC TAC TGC CGT CCA ATT	70
IGF-1	TCA CCA GCT CCA CCA CAG CTG GAC	71
Skeletal Actin	TGT GGC TAT CCA GGC GGT GCT GTC CCT	72
Cardiac Actin	ATC GTA TGC AAA AGG AAA TCA CTG CAC TG	73
VSM Actin	ACT TAG AAG CAT TTG CGG TGG ACG A	74
β Actin	CGG ACT CAT CGT ACT CCT GCT TGC TG	74
a MHC	TGA CCC GAG GCA AGC TCT CCT ACA	76
β MHC	CAG CGT TCT GTC AAT GAC CTC ACC AG	77
MLC2a	TGA TCC CAT CCC TGT TCT GGT CAA TG	78
MLP	AAG ACC TGC TTC CGC TGT GCC ATC TGT	79
TGF β1	CCG CGT GCT AAT GGT GGA CCG CAA CAA	80
Coll III	AGC CTT CTA CAC CTG CTC CTG TGC TTC	81
Fibronectin	CTG CTG AAC CCA GTC CCG ATG GCA CCA	82
CARP	CCG TCC GTT TAT ACT CAT CGC AGA C	83
GATA-4	CTG CCA GAC TAC CAC CAC CAC GCT G	84
GATA-6	CCA GAC GCC ACT GTG GAG ACG AGA C	85
SERCA 2	CAT CCG AGT TGG AAG TAC CAA GGT CCC	86
PLBN	AGT GCA ATA CCT CAC TCG CTC GGC TAT CA	87
MnSOD	AGA GCA GGC AGC AAT CTG TAA GCG ACC TT	88
MCAD	TGG CGT CCC TCA TCA GCT TCT CCA C	89
GAPDH	AAG CCC ATC ACC ATC TTC CAG GAG CGA GA	90

ANALYTICAL PCR. The Access RT-PCR System, using AMV reverse transcriptase and *Tfl* DNA polymerase, (Promega, Madison, WI) was used for RT-PCR. 50 µl RT-PCR reactions have 10 µl of AMV/*Tfl* 5x Reaction Buffer, 3 mM $MgSO_4$, 200 µM dNTPs, 600 nM ROX standard, 5 Units AMV Reverse Transcriptase, 5 Units of *Tfl* DNA polymerase, 200 nM probe, 100-300 nM primers and 100 ng of

total RNA. RT-PCR conditions are 45 minutes at 48°C, 2 minutes at 95°C, and 40 cycles of 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 72°C. Standard curves were obtained each time a gene was assayed using total RNA prepared from pooled adult C57BL/6 ventricles with 400 ng, 100 ng, 25 ng, 6.25 ng per reaction. The amount of test RNA may be reduced to 1 ng of total RNA for abundant genes or increased to 1 µg for rarer transcripts, with standard curves adjusted accordingly. Reactions were run in 96 well plates on a Model 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA) and results were analyzed using Sequence Detection Software (PE Applied Biosystems, Foster City, CA, USA).

CALCULATION OF RELATIVE RNA EQUIVALENTS. The equivalent amount of control RNA in each sample is calculated from a plot of Ct versus $\log_{10}$ [RNA] (µg/reaction) based on the control RNA dilution run for each gene. Since there is no absolute copy number, the quantity is relative to the pooled ventricular RNA used for the standard curve, the relative RNA equivalent. GAPDH was used as an internal control to normalize differences in input sample RNA. Each sample was run in a GAPDH assay at the beginning of the series of assays and then at the end. The diluted RNA can be stored at 4°C for up to three weeks to allow for assay completion, during which time there is no change in the GAPDH Ct value. The quantity of input RNA in each reaction was corrected to 100 ng based on the GAPDH standard curve.

STATISTICAL METHODS. Statistical parameters were calculated using Statview® software (Abacus Concepts, Inc., Berkeley, CA). Two-tailed *t* tests, assuming equal variance, were used for comparisons between two groups. For multiple groups, analysis of variance was utilized, with post hoc analysis with Scheffe's test. To minimize the effect of unequal variance among groups, the $\log_{10}$ of the RNA equivalent values was employed. A *p* value less than 0.05 was considered significant.

Example 1

Expression Analysis in Postnatal Myocardium

The purpose of this experiment was to use the quantitative reverse transcriptase-polymerase chain reaction assay disclosed herein to analyze cardiac gene expression.

The selection of genes studied was based on known differences in cardiac gene expression from models of cardiac hypertrophy in the mouse and other species, and also to test a number of candidate genes which may impact cardiac function (Table 2). Representative categories of cardiac genes were examined, including natriuretic peptides, embryonic contractile proteins,

Ca^{++}-regulatory proteins, mitochondrial enzymes, nuclear factors, matrix proteins, and signal transduction molecules.

Age- and sex-based effects on cardiac gene expression were examined in ventricular tissue from C57BL/6 mice. Body weight (BW) and ventricle weight (VW) were compared among groups of young adult (3 month old) and aged (18 month old) males and females (4 to 6 animals per group). VW or VW/BW weight was not different between groups (data not shown), reflecting proportional growth of the myocardium with increasing body weight. Of the 29 genes assayed only α-skeletal actin showed a difference between adult groups. Aged males had a higher level of α-skeletal actin mRNA compared to age matched females (3.5-fold, $p = 0.003$); this difference did not reach statistical significance in younger animals (2.8-fold, $p=0.064$).

Neonatal gene expression was determined from pools of 1 day-old ventricles and was compared to adult data (pooled from all individual measurements, representing both sexes and ages) to identify differences specifically associated with the postnatal myocardium, a tissue that reflects embryonic patterns of gene expression. mRNA expression levels for 7 genes showed no difference between neonate and adult myocardium, 15 were elevated in the postnatal ventricles and 3 were repressed compared to adults (Table 2).

Table 2. Gene expression in Developmental hypertrophy

Gene	Neonate	Adult	Neonate:Adult	p
ANP	463±92	77±60	6.1	<0.01
BNP **	260±127	67±3	3.9	<0.01
NPR-A	126±32	92±24	1.4	0.04
NPR-B	55±12	61±15	0.9	0.54
ET-1	165±46	69±36	2.4	<0.01
ETAR	226±59	85±26	2.7	<0.01
ACE **	38±12	94±35	0.4	0.01
AT1aR	56±7	64±24	0.9	0.54
PF2aR	21±5	100±45	0.2	0.01
VEGF	33±6	41±15	0.8	0.43
IGF-1	185±63	39±22	4.7	<0.01
α Sk Actin **	8.5±03	29±21	0.3	0.12
α Cardiac Actin	82±22	66±30	1.3	0.38
VSM Actin	452±71	55±22	8.3	<0.01
β Actin **	160±19	100±21	1.6	<0.01
α MHC	42±7	43±16	1.0	0.90
β MHC	579±63	38±23	15.1	<0.01
MLC2a	249±32	44±20	5.6	<0.01
MLP	37±7	51±28	0.7	0.41
TGF β1 **	143±18	78±22	1.9	<0.01
Fibronectin	586±141	86±54	6.8	<0.01
Collagen III	277±18	75±24	3.7	<0.01

CARP **	90±20	55±39	1.6	0.14
GATA - 4 **	128±26	89±29	1.4	0.03
GATA - 6 **	511±150	94±34	5.4	<0.01
SERCA	26±12	50±14	0.5	0.01
PLBN	48±7	65±38	0.7	0.45
MnSOD	54±12	70±43	0.8	0.54
MCAD	33±8	45±29	0.7	0.50

** 24 animals

Normalized RNA equivalent values were obtained from three pools of 22-28 ventricles from Day 1 neonates and 16 animals for the adult average except where noted. Values are the mean ± SD.

Example 2

Pressure Overload Hypertrophy

Gene expression analysis of mouse ventricles in response to acute POL by aortic constriction was performed in comparison to sham ventricles. In this mouse surgical model of cardiac hypertrophy there is an acute increase in VW/BW as the left ventricle undergoes concentric hypertrophy to normalize systolic wall stress. Increased mechanical load from aortic constriction is thought to be the primary stimulus underlying the hypertrophic response, however wall stress due to focal necrosis may also play a role. By one-week post-surgery there is a 30% increase in the VW/BW ratio of 12-week old female C57BL/6 mice (Table 3). mRNAs from a total of 20 genes were identified as induced or repressed (Table 4).

Table 3. Cardiac Hypertrophy in Pressure Overload

Condition	n	BW (g)	LVW (mg)	LVW/BW
Sham	6	32.3±2.3	114±12	3.5±0.4
POL	6	33.0±2.4	153±15	4.6±0.4
p		0.59	<0.01	<0.01

Table 4.
Differential Gene Expression in
Pressure Overload Hypertrophy (POL)

Gene	POL	Sham	POL/Sham	*p*
ANP	670±323	95±33	7.1	<0.01
BNP	293±61	79±43	3.7	<0.01
NPR-B	65±14	89±21	0.7	0.04
ACE	125±50	76±11	1.6	0.04
AT1aR	42±9	85±24	0.5	<0.01
PF2aR	39±12	63±21	0.6	0.04
IGF-1	110±20	60±10	1.8	<0.01
α Sk Actin	221±65	39±15	5.7	<0.01
β Actin	146±26	84±16	1.7	<0.01
α MHC	52±20	84±17	0.6	0.01
β MHC	189±78	73±20	2.6	<0.01
MLC2 α	22±9	38±13	0.6	0.04

Fibronectin	459±275	66±18	7.0	<0.01
Collagen III	338±153	50±18	6.8	<0.01
CARP	233±59	89±24	2.6	<0.01
GATA - 6	59±12	92±24	0.6	0.01
SERCA	37±14	73±20	0.5	<0.01
PLBN	37±23	80±20	0.5	<0.01
MnSOD	51±19	90±13	0.6	<0.01
MCAD	38±22	74±20	0.5	0.01

Values are the mean ± SD. No statistically significant changes (*p* value in brackets) were found for NPR-A (0.29), ET-1 (0.67), ETAR (0.54), VEGF (0.64), a Cardiac Actin (0.09), VSM Actin (0.39), MLP (0.55), TGFβ1 (0.12), and GATA-4 (0.51).

For direct comparison of neonatal and adaptive hypertrophy, statistically significant gene expression differences (Tables 2 and 4) are graphed together in a mirror-image plot (FIG. 2A and FIG. 2B). Genes are listed on the left , and values are taken from data in Tables 2 and 4. Ratios that are not statistically significant are given a value of one for comparative purposes. All genes that showed a significant change in either case are plotted.

These data demonstrate that the method disclosed and claimed herein can be used to quantitate gene expression and to quantitatively measure changes in gene expression with small amounts of total RNA. The ability to make such quantitative measurements rapidly allows the assay to be used to quantitate expression of a panel of genes in an array of samples.

Accordingly, a method for determining the level of expression of a gene is provided herein. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

We claim:

1. A method for determining a quantitative measure of the expression of a gene of interest in a biological sample by determining a normalized RNA equivalent for the gene of interest in the sample, comprising:

(a) preparing a standard curve for the gene of interest using a reverse transcriptase-5'-exonuclease polymerase chain reaction (RT-PCR) by a method comprising

(i) preparing a reverse transcript standard dilution series from a total RNA extract having a known total RNA concentration,

(ii) amplifying each member of the reverse transcript standard dilution series using a 5'-exonuclease PCR assay, wherein the components of the assay comprise a forward primer, a reverse primer, a hybridization probe and a thermostable DNA polymerase, and wherein the primers and the probe are capable of specifically hybridizing to and forming a stable hybrid duplex with a segment of the reverse transcript of mRNA encoded by the gene of interest, or its complement, the primers are capable of priming an extension reaction, and the DNA polymerase is capable of catalyzing a primer extension reaction and has 5'-exonuclease activity,

(iii) monitoring fluorescence intensity from the 5'-exonuclease PCR assay in real time during each cycle of the PCR for each member of the reverse transcript standard dilution series,

(iv) determining a threshold cycle (Ct) for each member of the reverse transcript standard dilution series,

(v) determining an RNA equivalent from a plot of Ct versus the log of the amount of total RNA in each member of the reverse transcript standard dilution series, and

(vi) normalizing the RNA equivalent to provide a normalized RNA equivalent standard curve for the gene of interest;

(b) assaying the sample in the RT-PCR and determining the normalized RNA equivalent for the gene of interest in the sample from the standard curve prepared in step (a).

2. The method of claim 1, wherein the normalizing of step (a)(vi) is based on the known RNA concentration of the total RNA extract and on a plot of Ct determined for a housekeeping gene versus the log of the amount of total RNA in each member of the reverse transcript dilution series.

3. The method of claim 1, wherein the reverse transcript standard dilution series is prepared by reverse transcribing the total RNA extract to provide a total RNA reverse transcript and diluting the total RNA reverse transcript.

4. The method of claim 1, wherein the reverse transcript standard dilution series is prepared by diluting the total RNA extract to provide a total RNA extract dilution series and reverse transcribing the total RNA extract dilution series.

5. The method of claim 1, wherein the total RNA extract is a total RNA extract of the biological sample.

6. A method for determining the effect of a treatment on a quantitative measure of the expression of a gene of interest in sample by determining a normalized RNA equivalent for the gene interest in a first untreated sample and a second treated sample, comprising:

(a) preparing a standard curve for the gene of interest using a reverse transcriptase-5'-exonuclease polymerase chain reaction (RT-PCR) by a method comprising

(i) preparing a reverse transcript standard dilution series from a total RNA extract having a known total RNA concentration,

(ii) amplifying each member of the reverse transcript standard dilution series using a 5'-exonuclease PCR assay, wherein the components of the assay comprise a forward primer, a reverse primer, a hybridization probe and a thermostable DNA polymerase, and wherein the primers and the probe are capable of specifically hybridizing to and forming a stable hybrid duplex with a segment of the reverse transcript of mRNA encoded by the gene of interest, or its complement, the primers are capable of priming an extension reaction, and the DNA polymerase is capable of catalyzing a primer extension reaction and has 5'-exonuclease activity,

(iii) monitoring fluorescence intensity from the 5'-exonuclease PCR assay in real time during each cycle of the PCR for each member of the reverse transcript standard dilution series,

(iv) determining a threshold cycle (Ct) for each member of the reverse transcript standard dilution series,

(v) determining an RNA equivalent from a plot of Ct versus the log of the amount of total RNA in each member of the reverse transcript standard dilution series, and

(vi) normalizing the RNA equivalent to provide a normalized RNA equivalent standard curve for the gene of interest; and

(b) assaying the first and second unknown samples in the RT-PCR and determining the normalized RNA equivalent for the gene of interest in the first and second unknown samples from the standard curve prepared in step (a).

7. The method of claim 6, wherein the normalizing of step (a)(vi) is based on the known RNA concentration of the total RNA extract and on a plot of Ct determined for a housekeeping gene versus the log of the amount of total RNA in each member of the reverse transcript dilution series.

8. The method of claim 6, wherein the reverse transcript standard dilution series is prepared by reverse transcribing the total RNA extract to provide a total RNA reverse transcript and diluting the total RNA reverse transcript.

9. The method of claim 6, wherein the reverse transcript standard dilution series is prepared by diluting the total RNA extract to provide a total RNA extract dilution series and reverse transcribing the total RNA extract dilution series.

10. The method of claim 6, wherein the total RNA extract is a total RNA extract of the biological sample.

11. The method of claim 6, wherein the treatment is selected from the group consisting of physiological treatment, a pharmacological treatment, onset of a pathological state, a surgical treatment, a genetic treatment, combinations thereof, and an adaptive response thereto.

12. A method of determining a quantitative measure of the expression of a panel of genes of interest in sample by determining a normalized RNA equivalent for each gene of interest in the sample, comprising:

(a) preparing a standard curve for each gene of interest using a reverse transcriptase-5'-exonuclease polymerase chain reaction (RT-PCR) by a method comprising

(i) preparing a reverse transcript standard dilution series from a total RNA extract having a known total RNA concentration,

(ii) amplifying each member of the reverse transcript standard dilution series using a 5'-exonuclease PCR assay, wherein the components of the assay comprise a forward primer, a reverse primer, a hybridization probe and a thermostable DNA polymerase, and wherein the primers and the probe are capable of specifically hybridizing to and forming a stable hybrid duplex with a segment of the reverse transcript of mRNA encoded by the gene of interest, or its complement, the primers are capable of priming an extension reaction, and the DNA polymerase is capable of catalyzing a primer extension reaction and has 5'-exonuclease activity,

(iii) monitoring fluorescence intensity from the 5'-exonuclease PCR assay in real time during each cycle of the PCR for each member (iv) determining a threshold cycle (Ct) for each member of the reverse transcript standard dilution series,

(v) determining an RNA equivalent from a plot of Ct versus the log of the amount of total RNA in each member of the reverse transcript standard dilution series, and

(vi) normalizing the RNA equivalent to provide a normalized RNA equivalent standard curve for the gene of interest;

(b) assaying the sample in the RT-PCR using the primers and probe for each gene of interest and determining the normalized RNA equivalent for each gene of interest in the sample from the standard curves prepared in step (a).

13. The method of claim 12, wherein the normalizing of step (a)(vi) is based on the known RNA concentration of the total RNA extract and on a plot of Ct determined for a housekeeping gene versus the log of the amount of total RNA in each member of the reverse transcript dilution series.

14. The method of claim 12, wherein the reverse transcript standard dilution series is prepared by reverse transcribing the total RNA extract to provide a total RNA reverse transcript and diluting the total RNA reverse transcript.

15. The method of claim 12, wherein the reverse transcript standard dilution series is prepared by diluting the total RNA extract to provide a total RNA extract dilution series and reverse transcribing the total RNA extract dilution series.

16. The method of claim 12, wherein the total RNA extract is a total RNA extract of the biological sample.

17. A method of determining the effect of a treatment on a quantitative measure of the expression of a panel of genes of interest in sample by determining a normalized RNA equivalent for each gene of interest in a first untreated sample and a second treated sample, comprising:

(a) preparing a standard curve for each gene of interest using a reverse transcriptase-5'-exonuclease polymerase chain reaction (RT-PCR) comprising

(i) preparing a reverse transcript standard dilution series from a total RNA extract having a known total RNA concentration,

(ii) amplifying each member of the reverse transcript standard dilution series using a 5'-exonuclease PCR assay, wherein the components of the assay comprise a forward primer, a reverse primer, a hybridization probe and a thermostable DNA polymerase, and wherein the primers and the probe are capable of specifically hybridizing to and forming a stable hybrid duplex with a segment of the reverse transcript of mRNA encoded by the gene of interest, or its complement, the primers are capable of priming an extension reaction, and the DNA polymerase is capable of catalyzing a primer extension reaction and has 5'-exonuclease activity,

(iii) monitoring fluorescence intensity from the 5'-exonuclease PCR assay in real time during each cycle of the PCR for each member (iv) determining a threshold cycle (Ct) for each member of the reverse transcript standard dilution series,

(v) determining an RNA equivalent from a plot of Ct versus the log of the amount of total RNA in each member of the reverse transcript standard dilution series, and

(vi) normalizing the RNA equivalent to provide a normalized RNA equivalent standard curve for the gene of interest; and

(b) assaying the first and second unknown samples in the RT-PCR and determining the normalized RNA equivalent for the gene of interest in the first and second unknown samples from the standard curve prepared in step (a).

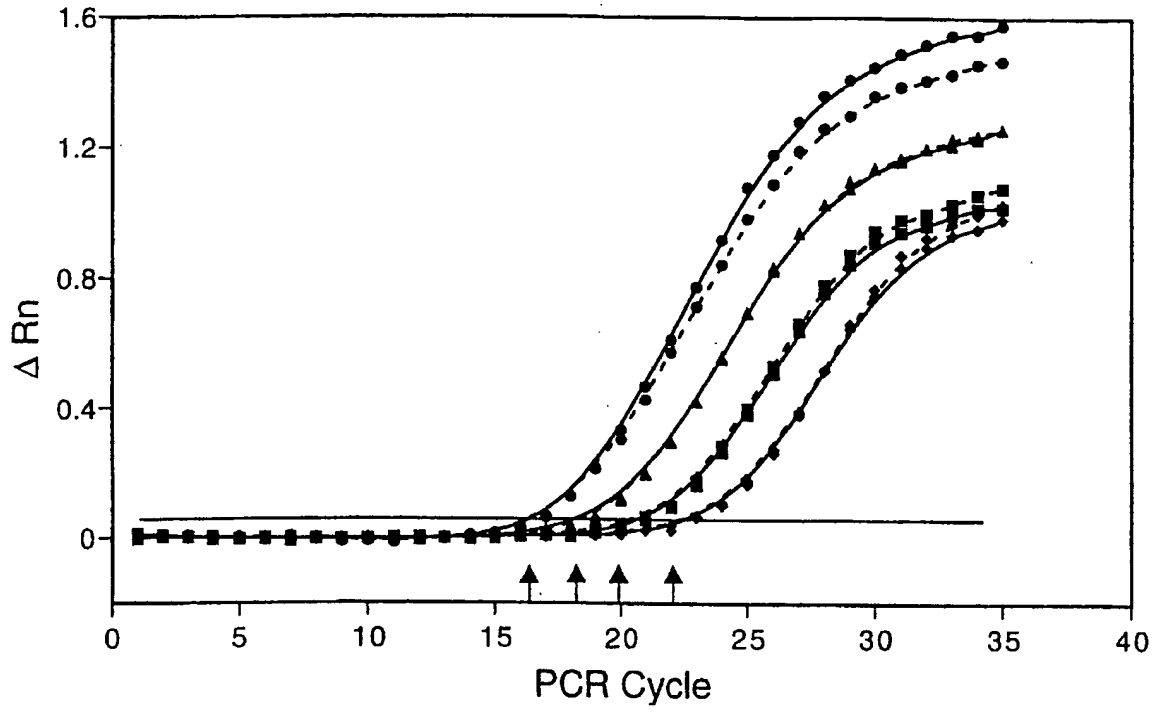
18. The method of claim 17, wherein the normalizing of step (a)(vi) is based on the known RNA concentration of the total RNA extract and on a plot of Ct determined for a housekeeping gene versus the log of the amount of total RNA in each member of the reverse transcript dilution series.

19. The method of claim 17, wherein the reverse transcript standard dilution series is prepared by reverse transcribing the total RNA extract to provide a total RNA reverse transcript and diluting the total RNA reverse transcript.

20. The method of claim 17, wherein the reverse transcript standard dilution series is prepared by diluting the total RNA extract to provide a total RNA extract dilution series and reverse transcribing the total RNA extract dilution series.

21. The method of claim 17, wherein the total RNA extract is a total RNA extract of the biological sample.

22. The method of claim 17, wherein the treatment is selected from the group consisting of physiological treatment, a pharmacological treatment, onset of a pathological state, a surgical treatment, a genetic treatment, combinations thereof, and an adaptive response thereto.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Δ Rn
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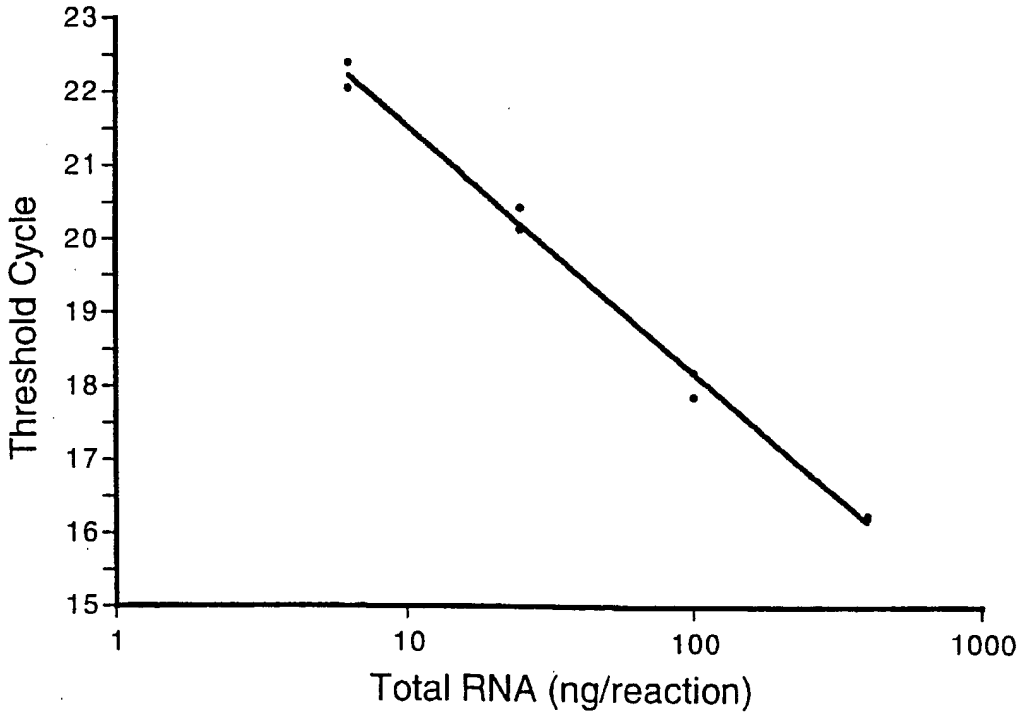
5
10
15
20
25
30
35
40
PCR Cycle

FIG._1A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19
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17
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15
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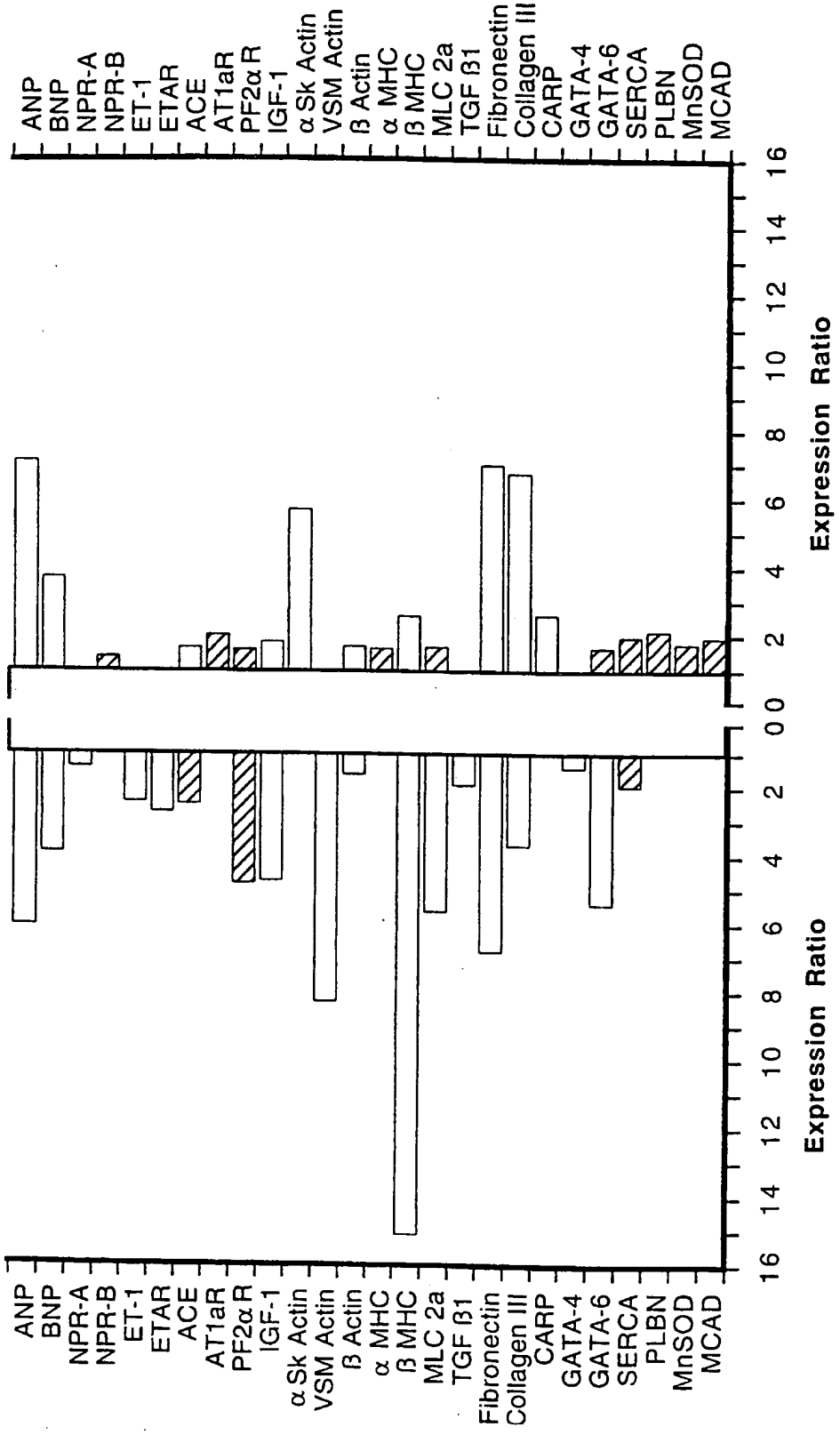
10
100
1000
Total RNA (ng/reaction)

FIG._1B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ETAR
ACE
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IGF-1
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β Actin
α MHC
β MHC
MLC 2a
TGF β1
Fibronectin
Collagen III
CARP
GATA-4
GATA-6
SERCA
PLBN
MnSOD
MCAD
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Expression Ratio

FIG._2B

FIG._2A

Sequence Listing

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